THE EFFECTS OF SHIFTING TEMPERATURE ON THE GROWTH OF *Listeria monocytogenes* AND *Salmonella* Typhimurium IN GOAT MILK SAMPLES COLLECTED FROM LOCAL DAIRY FARMS

by

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KESAN PERUBAHAN SUHU PADA PERTUMBUHAN *Listeria monocytogenes* **DAN** *Salmonella* Typhimurium **DALAM SAMPEL SUSU KAMBING DARI LADANG TENUSU TEMPATAN**

ABSTRAK

Objektif utama kajian ini adalah untuk menentukan keluk penyesuaian dan keluk kemandirian bagi *Listeria monocytogenes* dan *Salmonella* Typhimurium di dalam sampel susu kambing yang dikumpul dari ladang tenusu tempatan di Pulau Pinang. Kajian ini dijalankan bagi memahami kesan perubahan suhu ke atas *L. monocytogenes* and *S.* Typhimurium dari 37°C kepada beberapa suhu tekanan terpilih di dalam sampel susu kambing sebagai medium pertumbuhan. Kajian ini terbahagi kepada tiga bahagian. Bahagian pertama adalah untuk menentukan kehadiran mikroorganisma and patogen terutamanya *L. monocytogenes, S.* Typhimurium, kiraan plat total, kiraan kulat dan mold dan kiraan koliform di dalam susu kambing dari kedua-dua buah ladang tenusu. Bahagian kedua untuk menentukan profil pertumbuhan kedua-dua patogen, *L. monocytogenes* dan *S.* Typhimurium pada suhu 37°C dalam medium Trytone Soy Broth (TSB). Daripada keluk pertumbahan tersebut, fasa pegun (10^{-7}) pada 18 dan 10 jam pertumbuhan telah digunakan sebagai permulaan inokulum untuk keadaan perubahan suhu terpilih bagi *L. monocytogenes* and *S.* Typhimurium berikutnya. Untuk bahagian ketiga kajian, organisma tersebut dihidupkan pada suhu 37°C dalam medium TSB sebelum dipindahkan dalam susu kambing bermula dari suhu 0°C sehingga 90°C. Keputusan daripada pertumbuhan *L. monocytogenes* and *S.* Typhimurium yang dipindahkan dari suhu 37°C kepada suhu rendah (0°C sehingga 15°C) menunjukkan kesan bakteriostatik manakala perubahan dari suhu 37°C kepada suhu 25°C menunjukkan keupayaan *L. monocytogenes* dan *S.* Typhimurium untuk bertumbuh secara

perlahan-lahan di dalam sampel susu kambing. Sebaliknya, perubahan *L. monocytogenes* dan *S.* Typhimurium kepada suhu tinggi (50°C sehingga 70°C) memusnahkan kedua patogen selepas beberapa jam perubahan suhu dilakukan dan memunjukkan garis-lurus kinetik kematian. Namun demikian, keadaan 'toleransi terhadap suhu' hanya diperolehi bagi *S.* Typhimurium pada 50°C dalam sampel susu kambing. Namun demikian, tiada pertumbuhan kedua- dua pathogen dikenalpasti pada suhu bermula dari 75°C sehingga 90°C dalam susu kambing. Terdapat perbezaan yang signifikan ($P<0.05$) pada perubahan semua suhu dalam agar selective. Selain itu, *L. monocytogenes* dan *S.* Typhimurium didapati tercedera kesan daripada perubahan suhu dan ditunjukkan dalam imej SEM dan juga kiraan peratus kecederaan sel patogen. Data model pertumbuhan dan kemandiran *L. monocytogenes* dan *S.* Typhimurium menunjukan model Pertama adalah bersesuian dengan data experimen dengan jumlah error yang minimum. Daripada kajian yang dijalankan dapat dirumuskan bahawa penyesuaian dan kemandirian *L. monocytogenes* dan *S.* Typhimurium bergantung kepada jenis perubahan suhu dan medium pertumbuhan yang digunakan.

EFFECTS OF SHIFTING TEMPERATURE ON THE GROWTH OF *Listeria monocytogenes* **AND** *Salmonella* Typhimurium **IN GOAT MILK SAMPLES COLLECTED FROM LOCAL DAIRY FARMS**

ABSTRACT

The main objective of this study was to determine the adaptation and survival curves of *Listeria monocytogenes* and *Salmonella* Typhimurium in goat milk samples collected in local dairy farm in Penang Island. This research was conducted to understand the effects of shifting *L. monocytogenes* and *S.* Typhimurium from 37°C to various selected stress temperature in goat milk samples as growth media. The study was divided into 3 main parts. First part of the study was to determine the prevalence microorganisms and pathogens especially *L. monocytogenes, S.* Typhimurium, total plate count, yeast and mould count and coliform count in goat milk samples from 2 farms. The second part was to establish the growth profile for these two pathogens at 37°C in Tryone Soy Broth media. From the plotted growth curve, stationary phase at 10^{-7} CFU/ml at 18 and 10 hours of growth was used as the starting inoculum for the stress conditions for *L. monocytogenes* and *S.* Typhimurium, respectively. In the third part of study, these pathogens were grown at 37°C in TSB medium before shifting to goat milk at different temperature ranging from 0°C to 90°C. Results of shifting *L. monocytogenes* and *S.* Typhimurium from 37°C to lower temperatures from 0 up to 15°C showed bacteriostatic effects, while shifting from 37°C to 25°C showed the ability of *L. monocytogenes* and *S.* Typhimurium to grow gradually in goat milk. In contrast, shifting of *L. monocytogenes* and *S.* Typhimurium to higher temperatures (from 50°C to 70°C) exhibited growth of both pathogens after few hours of stress and demonstrated straight-line death kinetics. However, thermotolerance was observed only for *S.*

Typhimurium at 50°C in goat milk. While, no growth of both pathogens observed starting from 75°C to 90°C in goat milk sample. There were significant difference ($P<0.05$) of *L. monocytogenes* and *S.* Typhimurium growths at all the stress temperatures on selective agar. Furthermore, *L. monocytogenes* and *S.* Typhimurium were injured due to stress condition which was observed in the SEM images and also percentage of injury. Modeling the growth and survival of *L. monocytogenes* and *S.* Typhimurium shows that the primary model fits the experiment data by minimizing the error. The present study concluded that the adaptation and survival of *L. monocytogenes* and *S.* Typhimurium depends on the type of temperature stress and on media used.

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LIST OF ABBREVIATIONS

a_w	Water activity
a.m.	Ante meridiem (before noon)
CFU/ml	Colony forming Unit per milliliter
EC broth	Escherichia Coli broth
EMB agar	Eosin Methylene Blue Agar
MPN/ml	Most probable number per milliliter
PALCAM agar	Polymixin, Acriflavine,Lithium chloride, Ceftazidine, Aesculin Mannitol agar
p.m.	Post meridiem (after noon)
p.s.i	Pounds per Square Inch (Pressure Unit)
SD	Standard deviation
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
XLD agar	Xylose Lysine Deoxycholate agar
µm	Micrometer
°C	Degree Celsius

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CHAPTER 1

INTRODUCTION

1.1 Research background

Goat milk has played a tremendous role in providing health benefits to humans (Raynal-Ljutovac *et al.,* 2008; Silanikove *et al.,* 2010). It is being used in preparation of various food products, which includes beverages (fortified or flavored), fermented and frozen products (cheese, yogurt, ice cream), and sweets and candies (Ribeiro & Ribeiro, 2010).

In raw milk, bacterial contamination (growth and multiplication) can occur every half hour at 25ºC, pH range of 6.0-6.5 (Millogo *et al.,* 2010). The presence of pathogens in raw milk is necessary as a measure of food safety and awareness to consumer (Millogo *et al.,* 2010). In goat milk, bacteria (spoilage and pathogenic) can gain entry via infected goats with sub-clinical or clinical mastitis, environmental conditions prevailing in farms, utensils used for storage, transportation and processing environment.

Malaysia statistic showed that, 95% of goat breeding is done for meat and only 5% is used for milk production (Veterinary Department of Penang, 2011). Raw goat milk is an excellent source of nutrient (4.2% fat, 4.6% lactose, 3.25% protein, and 0.65% minerals) which favours the growth of pathogenic and spoilage bacteria such as *Bacillus cereus, Brucella melitensis, Clostridum botulinum,* Coliform that produce *Shiga toxin, L. monocytogene*s, *Salmonella sp., Escherichia coli 0157:H7,*

Streptococcus sp., and *Staphylococcus aureus* (Seifu *et al.*, 2004; Kousta *et al.*, 2010; Silanikove *et al.*, 2010).

Although fresh milk is regarded as sterile, bacteria are introduced into milk through infection conditions or environmental sources during milking and processing (Martins *et al.*, 2006). Studies on raw milk stored at refrigeration temperature have confirmed the presence of dominant microflora such as species of lactic acid bacteria (*Lactococcus* and *Lactobacillus* spp), *Pseudomonas* spp., the group *Micrococcaceae* (*Micrococcus* and *Staphylococcus* spp.), *Bacillus, Clostridium* and *Listeria* spp. and *Enterobacteriaceae* (Lafarge *et al.*, 2004) in milk. Microbiological quality of bulk tank milk showed the presence of gram-negative bacteria which was categorized into two groups, coliform and noncoliforms (Jayarao & Wang, 1999).

The contamination and/or cross-contamination of milk by spoilage and pathogenic microorganisms occur either during handling or during transportation and storage. Practising and establishing appropriate methods for reduction of microorganisms directly depends on adopting proper hazard analysis critical control point systems in the dairy industry (Claeys *et al.*, 2002; Millogo *et al.*, 2010). Temperature stress (heat or cold) is one of the vital environmental factors that can manage or inhibit the growth of microorganisms during various stages of processing (Fu *et al.*, 1991; Yousef & Carlstrom, 2003). Unfortunately, fluctuating high temperatures involved during various stages of milk processing can affect the overall nutritional quality, especially those of milk proteins and vitamins (Efighia *et al.*, 1997).

In developing countries, possible contamination of raw milk occurs either from contaminated hands, milk utensils (container), and udder or from the water used for cleaning. However, the contamination and cross-contamination can also occur in

developed regions of the world where hygienic practices are not practiced. Storing of fresh milk under unsuitable temperature and unhygienic conditions can lead to poor microbiological quality (Chye *et al.*, 2003; Lafarge *et al.*, 2004). Of late, food safety hazards associated with consumption of raw milk has been excellently reviewed by Oliver *et al.* (2009). Listeriosis outbreak was reported in contaminated milk and their products, meat and meat products, and in fresh produce (Sergelidis & Abrahim, 2009). While, worldwide, nearly 20 million cases of salmonellosis have been reported (*Salmonella* enterica serovars Typhi and Paratyphi) with 200,000 deaths (Boyle *et al.*, 2007).

In most of the food-borne illness cases, microbiological analysis is an important tool to determine the level of contamination of the food borne pathogens. However, in Malaysia, studies on the microbiological qualities of dairy and dairy products are lacking. The only published data on microbiological quality of cow milk in Malaysia was done by Chye *et al.* (2003). Therefore, this study was carried out to investigate in detail the growth and survival of food borne pathogen in goat milk under temperature stress condition.

In addition, some study also have reported that microorganisms directly respond to other stress conditions (such as toxic chemicals, heat, radiation, refrigeration, freezing, preservatives, and modified atmospheres) and suffer injury (Neidhardt & Van Bogelan, 2000; Trujillo et *al.*, 2002). Based on these, the major aim of this study was to investigate the effects of shifting temperatures (temperature abuse or stress temperature) on the survival and growth of *L. monocytogenes* and *S.* Typhimurium in raw goat milk.

Generally, in the tropical environmental conditions such as those encountered in Malaysia, raw goat milk can last between 7 and 8 h without refrigeration and can stay fresh for at least 5 days if refrigerated. However, reports available on the microbiological quality of goat milk or their products are scarce. Further, to our knowledge, since there have been no available reports on the effects of shifting temperatures on the survival of *L. monocytogenes* and *S.* Typhimurium in goat milk, this study evaluated the effects of various shifting temperatures on survival of *L. monocytogenes* and *S.* Typhimurium.

1.2 Problem statement

The shifting temperature range is an indication of the possible fluctuating temperatures encountered during various stages of food processing. The low temperature is an indication of refrigerated storage, ambient temperature is an indication of storing at room temperature, and high temperature is an indication of temperature danger zone in milk. It is envisaged that the results generated in this study will be useful to the dairy industries in order to predict the microbial risk assessment and provide an understanding on the behaviour of *L. monocytogenes* and *S.* Typhimurium during temperature abuse, particularly for goat milk.

As there are no established studies regarding the relationship between stress conditions and survival of pathogenic bacteria in goat milk in Malaysia, this study is the first documented one. There is a need to explain the survival of pathogens during different shifting temperature stress. Current practices of inspection carried out by Assistant Environmental Health Officers of the Ministry of Health at food premises (vendors/restaurants) are not sufficient to minimize the occurrence of foodborne illnesses. Combination of traditional microbiological analysis was conducted for

identification and enumeration of particular microbes in selected local goat farm. This research was carried out to obtain more knowledge on the microbiological quality of the subject at present.

1.3 Importance of studies

The importance of this study is that it provides the data on the microbial adaptation of *L. monocytogenes* and *S.* Typhimurium at different shifting temperatures in goat milk. The data were useful to understand the behavior of microorganisms during rapid changes occurring in temperatures at various stages of food preparation and processing.

Moreover, the results will be useful for the local authorities to conduct further research on microbiological quality and microbial adaptation of other microbes besides *L. monocytogenes* and *S.* Typhimurium to ensure safety of foods. The information will help the food industry to predict microbial risk assessment if microbial contamination occurs. In addition, the food industry will also understand the effects of stressed pathogens and their mechanisms on survival and pathogenicity during food preparation and processing.

The findings of this study would provide the fundamental knowledge for further studies on the mechanism of entry of microbes in dairy and dairy products in terms of physiological, biochemical and molecular aspects of microorganisms. The outcome of this study will help the government to provide guidelines on the preparation and handling of dairy and dairy products.

1.4 Objective of the study

1) To determine the prevalence of food-borne bacteria in goat milk from different farms.

2) To determine the growth curves of *L. monocytogenes* and *S.* Typhimurium at 37°C in TSB media.

3) To determine the growth rate constants and survival curves of *L. monocytogenes* and *S.* Typhimurium in goat milk from 2 different farms from 37°C to stress temperatures ranging from 0°C to 90°C .

4) To establish a predictive microbiological model on the survival of *L. monocytogenes* and *S.* Typhimurium in goat milk at different shifting temperatures.

CHAPTER 2

LITERATURE REVIEW

2.0 General

Nutrient contents in milk have comprehensive influence to human nutrition requirement, health protection, medical benefit and physiological function (Michaelidou, 2008; Ceballos *et al.,* 2009). Nutrient content in milk has been thoroughly reviewed and studied in different aspect to understand its benefits (Raynal- Ljutovac *et al.,* 2008; Barlowska *et al.,* 2011). Good quality, safe and affordable products can be produced for human consumption due to the high nutritive values of milk. However, common factors hindering these necessities are trading features, poor local infrastructure, product characteristic and lack of sanitary inspection during preparation (Carrasco *et al.,* 2011; Makita *et al.,* 2012).

Milk is the 'fluid synthesis' in mammary gland of mammals and is consumed by human for diverse reasons either raw or processed. Goat milk plays an important role in health and nutrition. It also has therapeutic potentials and product values. In general, goat milk is mild in color, with neutral pH, has appealing flavor, attractive odor and preferred taste by consumer. The chemical composition of goat milk allows the production of a variety products; fluid beverage products as low fat, fortified or flavored and UHT milk, fermented products; cheese, buttermilk and yogurt (Ribeiro & Ribeiro, 2010; Raynal-Ljutovac *et al.,* 2008). However, cow milk is one of the largest produced and consumed milk worldwide. Cow milk is an emulsion with small

fat or oil droplet which remains stable in surface active agent such as protein and lipids (Alarcon *et al.,* 2011; Raikos, 2010; Singh, 2011).

2.1 Milk, milk products and world production

Raw milk is processed into dairy products for different purposes and based on daily needs. Processing fresh milk (collected from animal's udder) into product requires heat treatment to control bacteria growth and prolonged product shelf life for marketing. Common processed dairy products are pasteurized or UHT liquid milk, cheese (milk in solid form with content of protein and fat), condensed milk (category of evaporated milk) and dried milk powder (Sieber, 2005; Ramirez *et al.,* 2006).

Studies were conducted in four countries in Europe (France, Germany, The Netherlands, and United Kingdom) on production of bulk dairy product from raw milk. The most demanding milk product manufactured from milk was cheese, whey powder and ice cream. However, reported study showed production of butter and non-fat milk powder has decreased in the last 15 years. Furthermore, trends of today's market are towards liquid low fat milk such as skimmed and semi-skimmed milk, ultra high temperature (UHT) milk, milk and milk drinks, fermented products and desserts (Ribeiro & Ribeiro, 2010).

According to the statistics by the Veterinary Department (2012), in Peninsular Malaysia, Sabah and Sarawak milk production from year 2001 to 2011 from livestock such as buffalo, cattle, goat and sheep was increasing yearly. Among these, states in Peninsular Malaysia were the largest producer followed by Sabah and Sarawak. Total milk produced from year 2001 to 2011 was 30.16, 35.96, 36.58,

38.77, 41.10, 45.45, 51.07, 56.49, 62.30, 67.00 and 70.87 million liters, respectively. While Table 2.1 shows the world milk production of year 2009.

Table 2.1: FOA databases for the year of 2009 shows the total world milk production as 696.6 million kg

Milk Type	Amount of milk produced	
	Million (kg)	%
Cow	580.5	83.3
Buffalo	90.3	13
Goat	15.1	2.2
Sheep	9	1.3
Camel	1.6	0.2

Barlowska *et al.,* 2011

The study also stated that two major contributors of goat milk were India and Bangladesh (26.3%) followed by European nations as France (3.8%) and Greece (3.3%). Cow milk producers were The European Union, The United States of America, India and Russia with production of 148.1 million kg, 85.9 million kg, 45.1 million kg and 32.3 million kg, respectively. Worldwide buffalo milk producer were India (60.9 million kg) and Pakistan (21 million kg). Sheep milk producer were China (12.2%), Europe (8.7%), Turkey (8.2%), Romania (7.2%) and Italy (6.1%). Finally, camel milk producer were Somalia (54.4%), Ethiopia (11.9%), Mali (8.1%), Sudan (7.5%) and Saudi Arabia (5.6%) (Barlowska *et al.,* 2011).

Dubeuf *et al.* (2004) reported that in the year 2001, the world goat milk producers were Africa, Asia, Europe and America. Asia was the largest producer of goat milk and the statistic in 2009 showed that India was the highest compare to the others. Table 2.2 shows the comparison of world milk production in year 2001 and 2009.

Table 2.2 World goat milk production for the year 2001 compared with 2009

Statistical databases of year 2001 (Dubeuf *et al*. 2004)		Statistic databases of year 2009 (Barlowska *et al.* 2011)	
Country	Goat milk production (1000 tons)	Country	Goat milk production (%)
Africa	2764	India	26.3
Asia	7085	Bangladesh	14.3
Europe	2321	France	3.8
America	341	Greece	3.3
Oceania	No data		

2.2 Composition and physico-chemical characteristics of milk

Milk is produced from four different animal species; bovine, ewe, goat and buffalo commonly for human consumption. Study by Jandal (1996) showed goat milk is to be pure white in color compared to cow milk (yellowish) because of the carotene present. Further, goat milk has a stronger flavor and 'goaty' smell compared to sheep milk due to presence of free short-chain fatty acids caused by rough handling. Cow's milk is also slightly acidic while goat's milk is more alkaline because of the different arrangement of phosphates. Other comparisons in the composition of goat, cow, human and sheep milk are shown in Table 2.3.

Table 2.3 Comparative composition of milk in different species

Component	Goat	Sheep	Human	Cow
Fat %	3.80	7.62	3.67-4.70	3.67
Solid-non-fat (%)	8.68	10.33	8.90	9.02
Lactose (%)	4.08	3.7	6.92	4.78
Protein (%)	2.90	6.21	1.10	3.23
Casein (%)	2.47	5.16	0.40	2.63
Whey protein (%)	0.43	0.81	0.70	0.60
Total ash (%)	0.79	0.90	0.31	0.73
Ca (%)	0.194	0.160	0.042	0.184
P (%)	0.270	0.145	0.06	0.235
Cl (%)	0.154	0.270	0.060	0.105
Vitamin A (IUg^{-1} fat)	39.00	25.00	32.00	21.00
Vitamin B_1 (mg per 100 ml)	68.00	7.00	17.00	45.00
Vitamin B_{12} (mg per 100 ml)	210.00	36.00	26.00	159.00
Vitamin C (mg per 100 ml)	20.00	43.00	3.60	2.00
Vitamin D (IU g^{-1} fat)	0.70	ND	0.27	0.70
Energy (Cal. Per 100 ml)	70.00	ND	68.00	69.00

Source: Jandal (1996)

According to Park *et al.* (2007) mineral contents (Ca, P, Mg, K, and Cl) in goat and sheep milk, are comparatively higher than cow milk. At the same time, vitamin content is significantly higher due to conversion of β - carotene into Vitamin A with whiter milk as end product. Concentration of macro-minerals may not be different among the milk but this may vary according to breed, diet, individual animal, stage of lactation and status of udder's health. Table 2.4 shows the mineral and vitamin contents (amount in 100g) of goat, sheep, cow and human milk.

Table 2.4 Comparison of mineral and vitamin contents of goat, sheep, cow and human milk

Constituents (in 100g milk)	Goat	Sheep	Cow	Human
Mineral				
Ca (mg)	134	193	122	33
P (mg)	121	158	119	43
Mg (mg)	16	18	12	4
K (mg)	181	136	152	55
Na (mg)	41	44	58	15
Cl (mg)	150	160	100	60
S (mg)	28	29	32	14
Fe (mg)	0.07	0.08	0.08	0.20
Cu (mg)	0.05	0.04	0.06	0.06
Mn (mg)	0.032	0.007	0.02	0.07
Zn (mg)	0.56	0.57	0.53	0.38
I (mg)	0.022	0.020	0.021	0.007
Se (μg)	1.3	1.00	0.96	1.52
Al (mg)	n.a.	0.05-0.18	n.a.	0.06
Vitamin				
Vitamin A (IU)	18.5	146	126	190
Vitamin D (IU)	2.3	0.18μg	2.0	1.4
Thiamine (mg)	0.068	0.08	0.045	0.017
Riboflavin (mg)	0.21	0.376	0.16	0.02
Niacin (mg)	0.27	0.416	0.08	0.017
Pantothenic acid (mg)	0.31	0.408	0.32	0.20
Vitamin B_6 (mg)	0.046	0.08	0.042	0.011
Folic acid (μg)	1.0	5.0	5.0	5.5
Biotin (μg)	1.5	0.93	2.0	0.4
Vitamin B_{12} (μg)	0.065	0.712	0.357	0.03
Vitamin C (mg)	1.29	4.16	0.94	5.00

Source: Park *et al*. (2007)

According to Metz *et al.* (2009) biochemical composition of milk varies depending on the characteristics of the animal which include breed, lactation stage and diet of the animal. Whereby, in a study by Borkova and Snaselova (2005), example of casein was observed to be part of protein which differentiate in ewe,

buffalo, bovine (α_s-casein) and goat milk (β -Casein); 82, 87, 80 and 77 %, respectively.

Ceballos *et al.* (2009) reported that goat milk protein and fat are more digestible and tolerable than cow's milk. He added that it provides tremendous energy in many metabolic processes and combats metabolic diseases. A study by Lucas *et al.* (2008) on nutritional composition of product made from raw cow and goat milk (cheese) indicated that goat milk fat was high in C8:0, C10:0, C18: 2n-6, retinol and –tocopherol (>30%) but has less amounts of C6:0, C12:0 and C18;0 (from 8 to 25%) than cow milk fat. However, cow milk fat was rich in C4:0, trans-11 C18:1, cis-9, trans-11 C18:2, xanthophylls and carotene (>30%) and less in C14:0, C16:0 and C18:3*n*−3 (from 16 to 23%) as compared to goat milk.

According to Park *et al.* (2007), physical properties such as density and surface tension of goat milk is equivalent to cow milk but is lower in sheep milk. Goat milk and sheep milk has higher specific gravity, viscosity, titratable acidity but lower refractive index and freezing point than cow's milk. Furthermore, lactic acid percentage or acidity is relatively similar in goats and cow milk when compared to sheep milk. While, pH value ranged between 6.50 to 6.85 in all milk type. Table 2.5 shows the comparison of phsico-chemical characteristic of structure between goat, sheep and cow milk (Park *et al.*, 2007).

Table 2.5 Comparison of physico-chemical characteristic of lipid and micelle structure between goat, sheep and cow milk

Features	Goat milk	Sheep milk	Cow milk
Physic-chemical values			
Unsaponifiable matter of milk fat (%)	0.41 ± 0.02	N.A.	0.41 ± 0.02
Acid value	0.47 ± 0.02	0.22 – 0.25	0.48 ± 0.05
Iodine value	19 – 20	20 – 35	27.09 ± 1.26
Saponification value	228.6 ± 5.24	230 – 245	232.3 ± 7.61
Reichert Meissl value	19 – 20	25 – 31	25 – 33
Polenske value	1.80 ± 0.35	1.6 – 1.5	1.4 – 1.3
Fat globule diameter (μm)	3.49	3.30	4.55
Micelle structure			
Non- centrifuge casein (% of total casein)	8.7	N.A.	5.7
Average diameter (nm)	260	193	180
Hydration of micelle (g/g MS)	1.77	n.a.	1.9
Mineralization of micelle (g/ca/100 casein)	3.6	3.7	2.9

Source: Park *et al*. (2007); N.A. = not available

2.3 Prevalence of bacteria and pathogens in raw goat milk and milk product

Raw goat milk has been identified as common path for gastrointestinal infection pathogens such as *Salmonella spp*., *Campylobacter jejuni*, *L. monocytogenes* and *E. coli 0157:H7* and bacteria as *Yersinia enterocolitica* (Tamagnini *et al.*, 2005; Tamagnini *et al.*, 2008; Oliveira *et al.*, 2011). Among these pathogens, *Salmonella* serotype Typhimurium is the most common pathogen determined in unpasteurized raw milk and its product collected from bulk tank milk and milk filter (Veling *et al.*, 2002; Nielsen & Ersboll, 2005). Futher more, other related studies showed *L. monocytogenes* in particular and other pathogens; *E. coli, S. aureus* and *Brucella melitensis* were determined in raw goat milk sample (Saanen and South African), kept at 30ºC for 60 hour (Park *et al*., 2004; Seifu *et al.*, 2004; Schirru *et al*., 2012).

The application of phenotypical and molecular approach on darfiyeh cheese (fermented product); variety made from raw goat milk and ripened in goat's skin container revealed the presence of *Streptococcus thermophilus*, *Enterococcus*

faecium, Enterococcus durans, Enterococcus faecalis, Enterococcus malodoratus, group D *Streptococcus sp., Lactococcus lactis subsp. lactis* and *L. lactis subsp. cremoris, Lactobacillus plantarum, Lactobacillus curvatus, S. haemolyticus, E. coli* and *Clostridium sp.* or *Eubacterium tenue* in the product (Herreros *et al.,* 2003; Badis *et al*., 2004; Serhan *et al.,* 2009; Maragkoudakis *et al*., 2010).

Various Enterococci species have been isolated from goat milk contaminated with faecal matter during milking process. The identified species were *Enterococcus faecium, E. faecalis, E. hiriae, E. gallinarum, E. avium, E. durans, E. casseliflavus* and in sample collected from bulk tank were *E. faecium* and *E. faecalis* (Psoni *et al.,* 2006; Platero *et al*., 2009; Serhan *et al*., 2009).

Mastitis pathogens in goat milk lactation in Thessaloniki, Greece showed that total viable counts, *Staphylococci* or *Micrococci*, *Streptococci*, coliform and aerobic sporeformer increased accordingly throughout the year (Haenlein, 2004; Moroni *et al.,* 2004). While, milk sample tested for caprine mastitis and identified species were *Micrococcus spp.* and *Staphylococcus* species; namely *S. epidermidis, S. aureus, S. caprae, S. lentus, S. simulans, S. capitis, S. lugdunensis, S. xylosus, S. chromogenes, S. hominis, S. arlettae, S. warneri, S. sciuri,* and *S. saprophyticus* (White & Hinckley, 1999; Akinedeh *et al.,* 2008).

In addition, study and identification of *Helicobacter pylori* in the goat milk samples showed that this pathogen is responsible for serious illnesses such as gastric or duodenal ulcer, and MALToma to human (Gueneau *et al*., 2002; Papiez *et al*., 2003; Quaglia *et al*., 2008; Vale & Vitor, 2010). Some other related factors that may influence bacteria growth in goat milk are weather, nutrients and hygienic level. In Malaysia, reported study on the presence of prevalence bacteria in raw milk was conducted only in cow milk by Chye *et al*. (2003).

2.4 Factors affecting microbial growth in milk

2.4.1 Temperature

Temperature is one of the significant factors that contribute to a microorganisms growth in milk and dairy products. In general, milk temperature enhances growth of both pathogenic and non-pathogenic food-borne bacteria in farms, dairy processing units and at local market level. In certain conditions, factors such as electrical faults, lack of cooling strategies and milk storage may also cause the fluctuating temperatures in the farms (Cayre *et al.,* 2005; Millogo *et al.,* 2010).

Temperature of freshly collected milk ranges between 37 to 38ºC which enhances the growth of mesophilic microorganisms once exposed to optimum growing temperature. Meanwhile, raw milk treated at temperature above 70ºC provides safe and quality milk to consumer as high temperature destroys pathogenic and food spoilage bacteria (Taylor & Woonton, 2009; Millogo *et al.,* 2010). In the cheese making industry, milk is usually treated at pasteurization temperature in order to obtain desirable flavor, texture and products free from bacterial contamination (Rehman *et al.,* 2000; Rynne *et al.,* 2007). Therefore, in dairy industry, temperature is a major factor to be considered in order to produce safe consumable milk especially from microorganisms' growth and survival.

2.4.2 pH

Microorganisms grow best at pH around 7.0 (6.6 – 7.5) and a few grow at pH lower than 4.0. Microorganisms grow well in raw milk at a pH around 6.3-6.5 and in milk products such as butter, buttermilk, cream and cheese (American mild and cheddar) at pH 6.1- 6.4, 4.5, 6.5 and 4.9- 5.9, respectively. Two important aspects in respiring microbial cells are function of its enzymes and transport of nutrients into

the cell (Jay, 2000). The shelf-life of milk and milk products commonly depends on the pH value and is also an indicator of estimate bacterial growth.

In milk, pH is affected by temperature during processing. As temperature increases, milk pH decrease and activates mesophilic bacteria growth which causes severe health risks to human (Millogo *et al.*, 2010). Commonly, high pH value of raw milk (6.98) from local markets may be caused by feeding material, lactation stage and by the dilution of milk with water.

2.4.3 Water activity (a_w)

Moisture content is the amount of water retained in food and it is a good source for microorganism growth in environment. Water activity (a_w) parameter is defined as ratio of water vapor pressure of food substrate to the vapor pressure of pure water at the same temperature (Chinn *et al.*, 2008). The relationship between temperature and water activity on microorganism growth explains the ability of bacteria to grow at any temperature with either low or high a_w. Basically growth is reduced by lowered a_w and increased at high a_w at optimum temperature; as bacteria require higher a_w compared to fungi (Jay, 2000; Chinn *et al.*, 2008). Table 2.6 shows the water activity preferred by organisms for their growth in food.

Table 2.6 Minimum a_w values for microorganism growth in food

Organisms	a_w	Organisms	a_w
Groups		**Groups**	
Most spoilage bacteria	0.9	Halophilic bacteria	0.75
Most spoilage yeasts	0.88	Xerophilic molds	0.61
Most spoilage molds	0.80	Osmophilic yeast	0.61
Specific organisms		**Specific organisms**	
Clostridium botulinum, type E	0.97	*Candida scottii*	0.92
Pseudomonas spp.	0.97	*Trichosporon pullulans*	0.91
Acinetobacter spp.	0.96	*Candida zeylanoides*	0.90
Escherichia coli	0.96	*Geotrichum candidum*	0.90
Enterobacter aerogenes	0.95	*Trichothecium spp.*	0.90
Bacillus subtilis	0.95	*Byssochlamys nivea*	0.87
Clostridium botulinum, type A and B	0.94	*Staphylococcus aureus*	0.86
Candida utilis	0.94	*Alternaria citri*	0.84
Vibrio parahaemolyticus	0.94	*Penicillium patulum*	0.81
Botrytis cinerea	0.93	*Eurotium repens*	0.72
Rhizopus stolonifer	0.93	*Aspergillus glaucus*	0.70
Mucor spinosus	0.93	*Aspergillus conicus*	0.64
		Aspergillus echinulatus	0.62
		Zygosaccharomyces rouxii	0.61
		Xeromyces bisporus	

Source: Jay (2000)

2.4.4 Acid

The lipophilic nature of organic acids compared to inorganic acids provides the tendency to inhibit microorganism growth rates. Further, inhibition rates depend on the concentration and types of acid used (Lopez *et al.*, 2011). A study by Lehrke *et al.* (2011) on *L. mononcytogenes* acidic injuries showed ascorbic acid and citric acid to cause most injuries to this pathogen. At low temperature, bacteria are inhibited in citric acid while inhibition occurs at pH 4.0, 4.5 and 5.0 in hydrochloric acid, lactic acid and propionic acid, respectively. Antimicrobial activity was also found high in acetic acid followed by lactic acid, citric acid, malic acid however low in HCL (Sorrells *et al.*, 1989). Naturally present acidity in food may be able to inhibit most of the bacteria growth however since milk is considered as a neutral product. Artificial addition of acid into milk may restrain the growth and increase milk shelf-life however there is possibilities for the milk to curdle.

2.5 Microbial contamination in milk

2.5.1 Food-borne bacteria contamination

Milk can be contaminated by various types of microorganisms during milking process followed by production, transportation and storage in the dairy industries. Commonly, spoilage organisms are the reason for the undesirable characteristic change (off-flavors) and at the same time harmful to human (Ali *et al.,* 2003; Ercolini *et al.,* 2009). In Malaysia, a study on raw cow milk shows possible contamination and high bacteria count due to infected cow udders, unhygienic milking procedures or equipment, inferior microbiological quality of water used for cleaning utensils and animals, and poor storage conditions of the milk (Chye *et al.,* 2003; Temelli *et al.,* 2006).

In general raw milk contamination via food-borne pathogens are influenced by various sources such as farm size, number of animals on the farm, hygiene; farm managements practices, geographical location, and season (Kousta *et al.,* 2010; Rosengren *et al.,* 2010). Studies on pathogen contaminations in milk showed *L. monocytogenes* and *Salmonella* to be shed through faeces (Nightingale *et al.,* 2004; Giannino *et al.,* 2009). Studies in France have shown that from 2000 dairy farms evaluated, the main source of *L. monocytogenes* contamination in raw milk was through environment such as poor feed quality, insufficient housing, milking hygiene, storage and transport. Moreover, poor hygiene practices and inappropriate milking lines are other risk factors for bacterial contamination (Waak *et al.,* 2002; Borucki *et al*., 2004; Kessel *et al.,* 2004). The same condition was discovered on the quality of raw milk collected from four different geographical zones in Tadla, Morocco where the highest rate of contamination was at irrigated zones while low rate of contamination was at semipublic farms. Source of bacterial contamination are

transportation, milking and pre-storage conditions of the milk (Kells & Gilmour, 2004; Afif *et al.,* 2008; Doijad *et al.,* 2011).

Bacillus cereus spores contamination in raw milk was found to be higher during grazing period (summer) than winter because of dirt, soil, and teats (Bonfoh *et al.,* 2006; Zhou *et al*., 2008). Studies showed that during winter, contamination was through bedding material. *Bacillus* spores are able to survive pasteurization, germinate and grow in refrigeration temperature during storage. In addition, these spores are capable of germinating in dairy equipment such as silo tank and packaging machines to further contaminate milk (Noriega *et al*., 2003; Svensson *et al*., 2006; Reyes *et al*., 2007; Salustiano *et al.,* 2009). Analysis on possible aerobic spore-forming bacterial contamination in raw milk from organic and conventional dairy farms shows heat-resistance spore forming bacteria is *Bacillus* species and initial contamination are through soil, grass, faeces, feed and milking equipment (Slaghuis *et al*., 1997; Svensson *et al.,* 2006; Coorevits *et al*., 2008; Carlin *et al.,* 2010).

S. aureus contaminates raw milk through infected udder and skin with clinical or subclinical *Staphylococcal* mastitis (Normanno *et al*., 2007; VCE, 2010). *E. coli* can spread from animals though the milking machines, milking installations, applied milking practices and faeces (Alatossava & Alatossava, 2006; Donkor *et al.,* 2007; Giannino *et al*., 2009). *Enterococci* and *Lactobacilli* are microflora found in human and animal intestinal tracts. It cause environment contamination to urban sewage, water and soil receiving fertilizers and further transfer into food products from animals (Kagkli *et al.,* 2007; Franz *et al.,* 2011).

Pasteurized milk are commonly contaminated and spoiled by Gram-negative psychrotrophic bacteria or Gram-positive spore-forming bacteria such as *Pseudomonas*, Enterobacteriaceae and *Aeromonas* during filling retail packages. The

filling of retail packages is an open process where milk comes in contact with surrounding air and aerosols followed by water condensed on machines. Therefore, water in and around the filling machine was verified as a factor that enhanced aerosol formation and increased risks for bacterial contamination to the milk during packaging (Marchand *et al*., 2008; Raats *et al*., 2011).

Other studies by Kagkli *et al*. (2007) and Kousta *et al*. (2010) have shown that the sources of contamination in milk were from farm environment, milk from unhealthy udder, water from farm, farmer's calabash, farmer's can and vendor's cans. The common types of microorganisms present in the environment were aerobic, mesophilic, Enterobacteriaceae, *S. aureus*, yeast and mould. The study added that all types of microorganisms were present in vendor's can, but for the farm environment, only aerobic, mesophilic, yeast and moulds were determined. Contamination in milk occurs at every level since the milk is collected from animal udders and sometimes it also happens through the animal itself into the milk. The presence of rare bacteria in milk as *Helicobacter spp*. is uncommon. However, as the source and carrier of the bacteria is different at every level, many other bacteria may also be present.

2.6 Heat resistant bacteria

The most heat resistant bacteria studied have been *Enterococcus faecalis* and followed by pathogens *Shigella sonnei* biotype A, *E. coli 0157:H7* and non-pathogenic *E. coli O3:H6. Salmonella, Serratia marcescens, K. pneumonia and Aeromonas hydrophia* have minimal resistance capacities while *Pseudomonas aeruginosa* was found to be less heat resistant (Spinks *et al.*, 2006; Lenz *et al*., 2010).

Milk contaminated with Gram positive bacteria; *Salmonella spp.* and heat treated at 55ºC for 35 minute was able to protect from heat probably through its complex substances and heat sensitive protein in the cells. Water concentration, soluble carbohydrates (mainly sucrose), salts, pH, fats and proteins are among the other factors in the heating medium that affects thermal resistance. Among studied milk types, whole milk gives more protection towards bacteria from heat than any other type of milk tested (Spinks *et al*., 2006).

Temperature is a key factor to control the growth rate and generation time of microbes, however bacterial characteristic is the major aspect influencing the growth slope between high and low temperatures. *Pseudomonas* stored at 7ºC has a faster generation time than other bacteria and was used to monitor spoilage of food product (Ratkowsky *et al*., 1982). In milk, heat treatment at high-temperature, short-time (71.7ºC for 15 seconds) and high temperature long time is (62.8ºC for 30 minutes) are found to be suitable to reduce microbial count.

At 65ºC, little thermal resistance was demonstrated by many species with log reductions in concentration within seconds (Casadei *et al*., 2001; Spinks *et al*., 2006; Lenz *et al.*, 2010). The temperature range from 55 to 65ºC was critical to eliminate the enteric or pathogenic bacteria components. Table 2.7, shows the D-value of bacteria grown at 37ºC and exposed to heat treatment (Spinks *et al*., 2006).

Table 2.7 D-value of non-starved (N) and starved (S) bacteria grown at 37ºC and exposed to instantaneous heat treatment

	55ºC	55ºC	60ºC	60ºC	65ºC	65 ºC
	N	S	N	S	N	S
Enterococcus faecalis (non-haemolytic)	901(±26)	-	131(±12)	-	19(±1.2)	-
Enterococcus faecalis (haemolytic)	633(±88)	509(±28)	77(±8)	92(±5)	7(±1.2)	-
Escherichia coli O3:H6 (wild type)	401(±30)	225(±20)	51(±2)	41(±1)	< 2	3(±0.3)
Listeria monocytogenes	305(±67)	311(±15)	63(±8)	54(±2)	3(±1.0)	3(±0.2)
Escherichia coli O157:H7 (ATCC43895)	223(±24)	232(±21)	67(±7)	69(±4)	3(± 0.3)	3(±0.2)
Shigella sonnei biotype A	354(±32)	305(±18)	54(±5)	39(±5)	3(±0.3)	4(±0.3)
Pseudomonas aeruginosa (wild type)	304(±35)	116(±9)	49(±8)	45(±3)	5(±0.6)	< 2
Salmonella typhimurium (LT2)	77(±12)	34(±3)	4(±1)	6(±1)	< 2	< 2
Serratia marcescens (wild type)	71(±3)	-	10(±0.6)	-	< 2	-
Klebsiella pneumonia (ATCC 13883)	22(±3)	19(±2)	< 2	< 2	< 2	< 2

Source: Spinks *et al.* (2006)
N; Non-starved, S; Starved

2.7 Effect of sublethal heating and sublethal injury on thermotolerant bacteria at high and low stress temperature

Bacteria sublethal injury is a short term tolerance loss at a particular condition which may be due to food processing and handling methods, thermal treatments, refrigeration, freezing, drying and irradiation or exposure to preservatives, acidity or low water activity and may happen to all Gram negative and Gram positive bacteria, endospores, yeast, and molds (Mossel *et al*., 1995; Sorhaug, 2000; Saldana *et al.*, 2009).

Generally in bacteria, physical and chemical treatment involves the cell envelope and cytoplasmic membrane damage however for Gram-negative bacteria

the outer membrane is also injured in the treatment (Sorhaug, 2000; Saldana *et al.*, 2009). Thermotolerance is also known as heat resistant cells growth by number of cells when it is exposed to heat treatment. Bacteria growth at 35ºC and held at 42 to 48ºC before thermal process shows less count compared to the bacteria exposed to less heat. Whereby, as sublethal heating is high, the thermotolerance effect of cells is low therefore may not cause problem when food is consumed (Brun *et al.*, 2009; Ells *et al.*, 2009). Table 2.8 shows the changes of the cell as treated with different treatments and mechanism of recovery. While, Table 2.9 shows the bacteria cell damage and its repair mechanisms due to different thermal treatment as freezing, drying and heating (Ray, 1986).

Table 2.8 Cell manifestation injuries by different treatments and repairing mechanisms

	Observed in cells treated by		
	Freezing	Heating	Drying
A. Manifestation of injury			
1. Loss of cellular materials	+	+	+
2. Sensitive to selective agents	+	+	+
3. Activation of some enzymes	+	+	+
B. Sites of damage			
1. Some wall components	+	+	+
2. Cell membrane	+	+	+
3. Ribosomes and rRNA	+	+	+
4. Structural DNA	+	+	+
C. Repair mechanisms			
De novo synthesis			
1. Mucopeptide	-	-	-
2. rRNA	+	+	+
3. DNA	-	-	-
4. Protein	-	+	+
5. ATP	+	+	+
Reorganisation			
1. LPS in Gram-negative bacteria	+	NS	+
2. TA in Gram-positive bacteria	+	+	NS

Source: Ray (1986)

rRNA, ribosomal RNA; LPS, Lipopolysaccharide; TA, teichoic acid; NS: Not studied

Other study on Scott A strain of *L. monocytogenes* grown at 48ºC for 20 minutes resulted in a 2.3 fold increase in D values at 55ºC and heat resistant condition observed when the strain was grown at 48ºC for 60 minutes and introduced to 60ºC

(Linton *et al*., 1995; Modi *et al.*, 2000). The various processing methods in the food industry environment which involve high and low temperature are among the causes of sublethal bacteria cell injury (Miller *et al*., 2006; Lu *et al*., 2011).

Table 2.9 Damages to vegetative bacteria by physical/ chemical agents

Agent damaging and higher intensity concentration	At cell wall or cell wall synthesis	Membrane leakage	Protein or protein synthesis	RNA (ribosomes) RNA synthesis	DNA
Adverse physical influences					
Heating		+	+	+	+
Freezing		+		+	+
Drying	(+)	+		+	+
Freezing-drying	+	+		+	+
Gamma and ultraviolet irradiation	+	(+)	(+)		+
Change of pO_2		+			
Osmotic shock	+	+			
Deprivation of nutrients					
Starvation/ senescence				+	
Disinfectants					
Chlorine and iodine[sp]			+		
Phenols [sp]	+	+	+		
Quarternary ammonium compound [sp]		+			
Antibiotic / Chemotherapeutic					
Acriflavin					+
Actinomycin D [sp]			+	+	
Bactriracin, vancomycin	+	+			
Chloramphenicol [sp]			+	(+)	
Erythromycin			+	(+)	
Nalidixic acid		(+)			+
Food preservatives					
Benzoic acid		+			+
Natamycin		+			
Nisin		+			
Nitrite		+			+
Parabens [sp]		+	+	+	+
Sorbic acid			+		
Sulphites		+	+		

Source: Mossel *et al*. (1995)
major effect; (+), minor effect; (-) possible effect; sp- inhibition of spore germination or out growth

According to Perni *et al*. (2007) the importance of repairing injured cells are to eliminate or minimize multiplication of competing organisms. However the mechanism of injuries in vegetative cells and spores are different from each other where alterations of macromolecules in cell structures are different.